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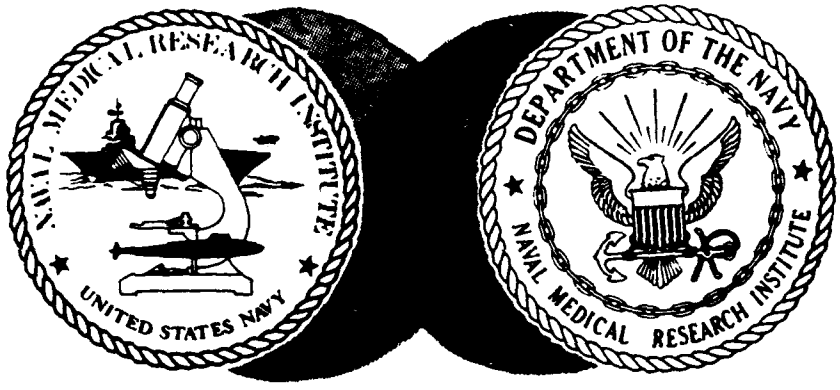


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PSEUDOMONAS AERUGINOSA TOXINS

O.R.PAVLOVSKIS AND B.WRETLIND

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During the last 30 years Gram-negative organisms have become the commonest cause of serious infections in hospitalized patients (Adler et al., 1970; McGowan et al., 1975). Infections caused by Pseudomonas aeruginosa have been particularly severe because a limited number of antibacterial agents are clinically effective in treatment and the patients who are most frequently involved are those whose defense mechanisms are seriously impaired. Normally commensal for humans, P. aeruginosa has been detected with increasing frequency in patients with serious disorders such as neoplastic diseases, cyctic fibrosis, burns, severe injuries, or in patients who have received immuno-suppressive therapy (Baltch and Griffin, 1977; Curtin et al., 1961; Fishman and Armstrong, 1972; Flick and Cluff, 1977; Forkner et al., 1958).

The pathological sequelae of these infections are not yet thoroughly understood. The pathogenicity of most Gram-negative bacteria has been attributed to endotoxin (LPS), but there is evidence that the endotoxin of P. aeruginosa is less toxic than that elaborated by other Gram-negative organisms. Moreover, P. aeruginosa does produce a number of toxic extracellular products, including exotoxin A, proteases, phospholipase, haemolysin, endotoxin, slime (Heckley, 1970; Liu, 1066b, 1974, 1979; Young and Armstrong, 1972). This chapter will review the more recent work in this field.

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# 3 *Pseudomonas aeruginosa* toxins

OLGERTS R. PAVLOVSKIS
and BENGT WRETLIND

## I. INTRODUCTION

During the last 30 years Gram-negative organisms have become the commonest cause of serious infections in hospitalized patients (Adler *et al.*, 1970; McGowan *et al.*, 1975). Infections caused by *Pseudomonas aeruginosa* have been particularly severe because a limited number of antibacterial agents are clinically effective in treatment and the patients who are most frequently involved are those whose defence mechanisms are seriously impaired. Normally commensal for humans, *P. aeruginosa* has been detected with increasing frequency in patients with serious disorders such as neoplastic diseases, cystic fibrosis, burns, severe injuries, or in patients who have received immunosuppressive therapy (Baltch and Griffin, 1977; Curtin *et al.*, 1961; Fishman and Armstrong, 1972; Flick and Cluff, 1977; Forkner *et al.*, 1958).

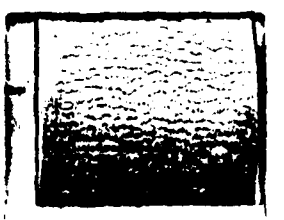
The pathological sequelae of these infections are not yet thoroughly understood. The pathogenicity of most Gram-negative bacteria has been attributed to endotoxin (LPS), but there is evidence that the endotoxin of *P. aeruginosa* is less toxic than that elaborated by other Gram-negative organisms. Moreover, *P. aeruginosa* does produce a number of toxic extracellular products, including exotoxin A, proteases, phospholipase, haemolysin, endotoxin, slime (Heckley, 1970; Liu, 1966b, 1974, 1979; Young and Armstrong, 1972). This chapter will review the more recent work in this field.

Because microbial products traditionally classified as toxins have been recognized as proteins, the term *toxin* will be restricted to proteins which possess properties harmful to the host (Bonventre, 1970; Bonventre *et al.*, 1967) (Table 1). The characteristics and biological activities of *P. aeruginosa* endotoxin and the common antigen (OEP) described by Homma and others

**Table 1** Extracellular proteins from *P. aeruginosa*

| Protein | MW×$10^{-3}$ | pI | $LD_{50}$ (mice) (μg) | Mode of action | Proposed role in pathogenicity |
|---|---|---|---|---|---|
| Exotoxin A | 54–71.5 | 5.0–5.1 | 0.06 | Ribosylation of ribosomal protein EF-2 | Inhibition of protein synthesis |
| Exoenzyme S | ND[a] | ND | ND | Ribosylation of ribosomal proteins | Possibly inhibition of protein synthesis |
| Elastase | 25–33–39.5 | 5.7–6.6 | 60–400 | Neutral metallo-proteinase | Tissue damage, degradation of coagulation and complement factors |
| Alkaline protease | 48 | 4.1–5.0 | 100–300 | Metalloproteinase | Probably same as for elastase |
| Phospholipase C[b] | ND | 5.75–5.9 | ND | Membrane damaging | Cytolytic, haemolytic, possibly destruction of lung surfactant |
| Leucocidin | 27 | 5.0–5.2 | 1 | Membrane damaging | Cytolytic, possibly induction of leucopenia |

[a] ND: No data.
[b] Synonyms: lecithinase, heat-labile haemolysin.



will not be discussed in any detail (Homma, 1968; Homma and Abe, 1972; Sasaki *et al.*, 1975; Tanato *et al.*, 1978, 1979).

## II. EXOTOXIN A

### A. General remarks

Liu and associates first demonstrated that extracellular products produced by the organism might contribute to the pathogenesis of the infection (Liu *et al.*, 1961). Detailed studies in this area were made possible by the isolation of a lethal, heat-labile exotoxin (Liu, 1966b). Injection of this exotoxin into dogs resulted in the haemodynamic and biochemical changes associated with shock and death (Atik *et al.*, 1968). These changes included acidosis, elevated levels of circulating catecholamines, an increased arterial–venous difference in oxygen saturation, circulatory collapse, and leucopenia. These observations were similar to those made in *Pseudomonas* infections. This work resulted in great stimulation of interest in the *Pseudomonas* toxin and its role in pathogenesis.

### B. Production, purification and characterization of exotoxin A

*P. aeruginosa* exotoxin A (toxin, lethal toxin), which is produced by over 90% of *P. aeruginosa* clinical isolates (Bjorn *et al.*, 1977; Pollack *et al.*, 1977; Sanai *et al.*, 1980), has been purified and characterized in several laboratories (Callahan, 1974, 1976; Iglewski and Sadoff, 1979; Leppla, 1976; Liu, 1973; Lory and Collier, 1980; Vasil *et al.*, 1976, 1977). *In vitro* it is secreted as a single peptide chain (Lory and Collier, 1980; Vasil *et al.*, 1977) containing four disulphide bridges and no free sulphydryl groups. The molecular weight has been estimated to be between 54 000 and 71 500 daltons (Callahan, 1974; Chung and Collier, 1977; Leppla, 1976; Lory and Collier, 1980; Vasil *et al.*, 1977) and pI 5.0–5.1 (Callahan, 1976; Leppla, 1976). As far as it is known, exotoxin A does not contain any unusual amino acids (Chung and Collier, 1977; Leppla, 1976) or components that would distinguish it from other non-toxic proteins.

Since nucleic acid derivatives found in laboratory media inhibit *in vitro* production of exotoxin A (Liu, 1973), trypticase soy broth dialysate (TSBD) plus glycerol and monosodium glutamate or modifications of this system have been used in most laboratories for exotoxin A production. *In vitro* exotoxin A production requires vigorous aeration, and its yields are decreased by high iron contents (50 $\mu g\ ml^{-1}$) (Bjorn *et al.*, 1979b). Recently a defined medium containing three amino acids (arginine, aspartic, alanine), a carbon source,

glycerol, and basal and trace salts have been used in our laboratory for exotoxin A production (DeBell, 1979; DeBell and Martin, 1979). Increasing amounts of exotoxin A were produced in cultures according to the following sequence: succinate ≥citrate >acetate >glucose >pyruvate. With 13 mM citrate, the amount of exotoxin A measured by enzyme activity and mouse lethality was approximately 90% of that produced in TSBD. A comparison of several strains of *P. aeruginosa* demonstrated that growth and exotoxin A production were essentially the same in the chemically defined medium as in TSBD. However, some strains of *Pseudomonas* do not produce good yields of exotoxin A when grown in chemically defined medium (Iglewski, personal communication). It should be noted that extracellular protease production was consistently lower in this medium. Since one of the problems encountered in large scale exotoxin A production is the proteolytic activity of the proteases produced by the organisms during growth, the chemically defined medium appears to be very well suited for exotoxin A production. Previously, in order to reduce the proteolytic activity either protease inhibitors such as nitrilotriacetate have been added to the growth medium (Callahan, 1976) or protease-deficient mutants such as the prototype strain PA 103 have been used (Atik *et al.*, 1968; Liu, 1979). A recent report, however, indicates that two of the proteases produced by *Pseudomonas*, alkaline protease and elastase, do not have any effect on exotoxin A enzymatic activity *in vitro*. Jagger *et al.* (1980) found no reduction in either biological or enzyme activity after treatment of exotoxin A with *Pseudomonas* proteases. Another bacterial protease, thermolysin, rapidly inactivated exotoxin A. The discrepancy between these results and the results of Sanai *et al.* (1980), who produced non-toxic fragments of exotoxin A following treatment with *Pseudomonas* elastase, is difficult to explain. It is possible that one group studied the native exotoxin, whereas the other group used a slightly denatured exotoxin with an altered configuration, thus rendering it sensitive to degradation by proteases.

The various procedures used for exotoxin A purification have been summarized by Iglewski and Sadoff (1979). Recently purification procedures using preparative polyacrylamide gel electrophoresis or affinity chromatography have been described (Callahan, 1976; Taylor and Pollack, 1978).

### C. Biological activity of exotoxin A

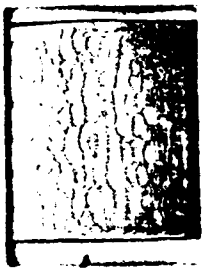
Exotoxin A is toxic to cells in culture (Middlebrook and Dorland, 1977; Pavlovskis, 1972; Pavlovskis and Gordon, 1972; Pavlovskis *et al.*, 1975) and to many animal species (Atik *et al.*, 1968; Liu, 1966b; Liu *et al.*, 1973; Pavlovskis and Shackelford, 1974; Pavlovskis *et al.*, 1975; Young and Pollack, 1979), including rhesus monkeys (Pavlovskis *et al.*, 1974a). On a weight basis and the total amount in culture fluids, exotoxin A is by far the most toxic *P.*

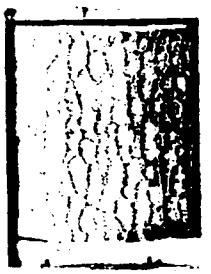
*aeruginosa* product tested to date. Its average mean lethal dose ($LD_{50}$) for a mouse is approximately 0.06 μg (Callahan, 1976), whereas the $LD_{50}$ for LPS is about 450 μg (Dyke and Berg, 1973) and for elastase 100 μg (Pavlovskis and Wretlind, 1979). Death usually occurs between 40 to 50 hours after an intravenous (IV) injection of exotoxin A (2 $LD_{50}$). With doses higher than 10–15 $LD_{50}$ survival time remains relatively constant—about eight hours (Pavlovskis *et al.*, 1975). Other animals tested (guinea pigs, rabbits, dogs) were as sensitive to exotoxin A as mice on body weight basis. A single intravenous injection (2 $LD_{50}$) of purified exotoxin A into mice elicited necrosis, cellular swelling, and fatty change in the liver within 4–8 hours and near total hepatocellular necrosis at 48 hours (Pavlovskis *et al.*, 1976). Frequently oedematous and haemorrhagic lungs and tubular necrosis and haemorrhages in kidneys have also been observed in exotoxin-A-treated mice and dogs (Liu, 1974). Hepatic necrosis is accompanied by a parallel rise in serum levels of aspartate and alanine aminotransferases (SGOT and SGPT) and alkaline phosphatase (Pavlovskis *et al.*, 1976). Following the IV injection of exotoxin A (2 $LD_{50}$) a rapid and significant decrease of protein synthesis occurs in mouse tissues (Pavlovskis and Shackelford, 1974). During the 2–4 hour interval following toxin administration there was greater than 50% inhibition in the liver and by 16–18 hours protein synthesis was reduced to less than 20% of that of the controls (untreated mice). In kidneys, spleen, and pancreas a 50% decrease in protein synthesis was noted 18 hours post-injection. As the animals approached death, protein synthesis decreases in every tissue examined. When mice were injected with doses of exotoxin A resulting in 75% mortality, a decrease in both IgM and IgG antibodies against sheep red blood cells could be demonstrated by the Jerne plaque assay in the surviving mice (Pavlovskis *et al.*, 1980).

The first indication of the mode of action of exotoxin A was provided by Pavlovskis and associates, who demonstrated that exotoxin A inhibits amino acid uptake in cultured cells and inhibits protein synthesis in exotoxin-treated mice (Pavlovskis and Gordon, 1972; Pavlovskis and Shackelford, 1974). Later Iglewski and Kabat (1975) showed that exotoxin A inhibits protein synthesis in a manner identical to diphtheria toxin fragment A. The inhibition requires nicotinamide adenine dinucleotide (NAD) and results in a block at an elongation step of polypeptide assembly. Exotoxin A catalyses the transfer of the adenosine-5′-diphosphate-ribosyl (ADP-ribose) moiety of NAD on to elongation factor 2 (EF-2) resulting in the inactivation of EF-2 (Iglewski and Kabat, 1975; Iglewski *et al.*, 1977b):

$$NAD^- + \text{EF-2} \xrightleftharpoons{\text{exotoxin A}} \text{ADP-ribose-EF-2} + \text{nicotinamide} + H^+.$$

Iglewski and associates have also demonstrated that the reaction is reversible and that the reverse reaction is favoured by low pH (Iglewski *et al.*, 1977b).



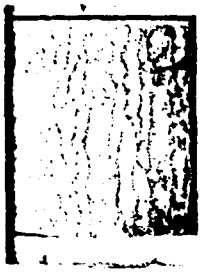


The transfer of the ADP-ribose moiety is to the same site on EF-2 in the case of both exotoxin A and diphtheria toxin, thus the reaction catalysed by one toxin can be reversed by the other (Chung and Collier, 1977; Iglewski *et al.*, 1977b). In the case of the diphtheria toxin the ADP-ribose is attached to EF-2 via an unusual amino acid – diphthamide (Brown and Bodley, 1979; Van Ness *et al.*, 1978). Diphthamide appears to be a derivative of histidine and is found in all EF-2 molecules examined (Brown and Bodley, 1978). It is not yet known whether this also occurs in the case of exotoxin A.

The two toxins, exotoxin A and diphtheria toxin, are distinct serologically (Iglewski and Kabat, 1975; Leppla, 1976) and exhibit different cellular specificites (Middlebrook and Dorland, 1977; Pappenheimer and Gill, 1973; Pavlovskis *et al.*, 1975; Pavlovskis and Gordon, 1972). Studies with cells in culture using both biochemical and electron microscopy methods suggest that even though both exotoxin A and diphtherial toxins are internalized via receptor mediated endocytosis, different receptors are probably involved (Dorland *et al.*, 1979; FitzGerald *et al.*, 1980; Leppla *et al.*, 1981; Middlebrook *et al.*, 1980; Vasil and Iglewski, 1978). Furthermore, experiments with non-toxic diphtherial protein (CRM 197), which cross-reacts serologically with diphtheria toxin, showed that while CRM 197 could effectively compete with diphtheria toxin and block its toxic action, the protein had no effect on the activity of exotoxin A (Vasil and Iglewski, 1978). Recently, a mutant strain of PAO-1 has been isolated which produces a protein immunologically indistinguishable from native exotoxin A, but is nontoxic for cultured Chinese hamster ovary cells (Cryz *et al.*, 1980). The protein (CRM) also has no ADP-ribosylating activity. Cross-reactivity studies with CRM and diphtheria toxin or CRM 197 have not been reported.

*In vitro*, exotoxin A is released as a proenzyme in an enzymatically inactive form. Enzymatic activity may be effected by reduction of the disulphide bonds in the presence of urea (Leppla *et al.*, 1978a; Lory and Collier, 1980; Vasil and Iglewski, 1976) or mild proteolysis of the exotoxin A molecule by *Pseudomonas* proteases in culture medium (Chung and Collier, 1977; Vasil *et al.*, 1977). Fragments ($M_r$, 26 000) which inhibit enzymatic activity have been isolated from culture fluids and some preparations of purified exotoxin A after storage (Chung and Collier, 1977; Vasil *et al.*, 1977). As the enzymatic activity of exotoxin A increases, its toxicity to cells in culture and mice decreases (Vasil *et al.*, 1977). It appears that the lethal activity of exotoxin A depends upon the intact molecule, whereas the enzymatic activity requires structural rearrangement.

Recently, Lory and Collier (1980) were able to produce and isolate enzymatically active fragments by cleaving full or partially reduced exotoxin A molecules by proteolytic or chemical methods. Incubation of reduced toxin with chymotrypsin in the presence of oxidized NAD yielded an enzymatically



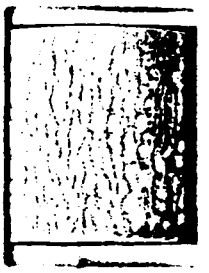
active peptide ($M_r$ 26 000). Treatment of toxin with CNBr or 2-nitro-5-thiocyanobenzoate yielded enzymatic fragments of $M_r$ 50 000 and 30 000 respectively. Enzymatically active non-cytotoxic fragments ($M_r$ 48 000) have also been isolated *in vitro* by treating exotoxin A with *P. aeruginosa* elastase followed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Sanai *et al.*, 1980).

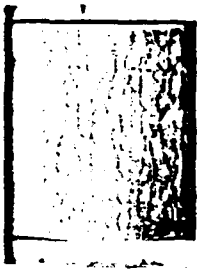
The lack of toxicity of the enzymatically active fragments implies that the remaining part of the exotoxin A molecule serves a function analogous to that of fragment B of diphtheria toxin. Thus exotoxin A resembles the structure–activity relationship of other bacterial toxins. That is, the molecule consists of an enzymatically active effector moiety ($M_r$ 26 000) and a moiety responsible for binding the toxin to the receptors on the cell surface. A receptor-binding fragment comparable to that of fragment B of diphtheria toxin has not yet been generated and isolated from purified exotoxin A, but such a fragment has been demonstrated in culture fluids (Vasil *et al.*, 1977). Thus isolation of a binding fragment may be only a technical problem in finding the appropriate conditions for the isolation and purification (Lory and Collier, 1980; Vasil *et al.*, 1977). It should be noted that although there are catalytic similarities between the enzymatic fragments of exotoxin A and diphtheria toxin ($M_r$ 21 145), there is no immunological cross-reactivity and they possess different pH and thermal stabilities (Lory and Collier, 1980).

To summarize, the evidence indicates that in native exotoxin A the enzymatic moiety is "buried" or distorted and that alterations in structure permit the enzymatic site to become exposed or to assume an active configuration. It is not known whether reduction or proteolytic processing of the toxin or both occur *in vivo* during the course of exotoxin A action. Although reduction of the molecule may possibly take place within the reducing environment in the cell, proteolytic processing may not only occur within the cell but also could take place prior to or after attachment to the cell receptor.

## D. The pathogenic role of exotoxin A

Although the biological activity of purified exotoxin A in cells is well documented in culture and in animals, the pathogenic role of exotoxin A in human disease is not clear. Several lines of evidence, however, strongly suggest that exotoxin A contributes to the pathogenicity of the organism. It has been shown that exotoxin A, the most toxic product of the organism, is produced *in vivo* in experimental animal infections (Pavlovskis *et al.*, 1977; Saelinger *et al.*, 1977; Stieritz and Holder, 1978), as well as in human patients in sufficient amounts to elicit antibody production (Cross *et al.*, 1981; Pollack *et al.*, 1976; Pollack and Young, 1979). Mice infected with toxigenic *P. aeruginosa* strains





have significantly reduced active EF-2 levels in the liver, whereas EF-2 levels were normal in infected mice pretreated with antitoxin (Pavlovskis *et al.*, 1978; Snell *et al.*, 1978) or mice infected with nontoxigenic strains (Pavlovskis *et al.*, 1978). Microscopic examinations of liver tissues from infected mice frequently revealed hepatocellular necrosis (Pavlovskis *et al.*, 1977; Wretlind and Kronevi, 1978) similar to that seen in mice injected with purified exotoxin A (Pavlovskis *et al.*, 1976). No analogous lesions were seen in mice pretreated with antitoxin. Ohman *et al.* (1980a), using nontoxigenic *Pseudomonas* mutants, has shown that exotoxin A production enhances the severity of experimental corneal infections in mice. Work in our laboratory (Pavlovskis *et al.*, 1977 and unpublished data) has produced similar results. The $LD_{50}$ in the experimental burned mouse infection model described by Stieritz and Holder (1975) was significantly higher for nontoxigenic mutants than their corresponding toxigenic parent strains (Table 2).

**Table 2** Virulence of exotoxin-A-producing *P. aeruginosa* strains and their toxin-deficient mutants[a] in mouse burn infections

| Strain | Toxin | $Log_{10}$ $LD_{50}$±SD[b] |
|---|---|---|
| PA 103 | + | 4.4±0.9 |
| PA 103-29 | − | 5.5±0.4 |
| PAO1 | + | 4.1±0.1 |
| PAO1-T1 | − | 5.8±1.0 |

[a] PA 103-29 Isolated and characterized: Ohman *et al.* (1980c); PAO1-T1: Ohman *et al.* (1980a).
[b] $P<0.02$.

The most convincing data regarding the pathogenic role of exotoxin A comes from immunization studies. Work in several laboratories (Liu and Hsieh, 1973; Pavlovskis *et al.*, 1977; Snell *et al.*, 1978) has demonstrated that passive immunization of mice with monospecific antitoxin resulted in significant increase in survival of mice infected with toxigenic strains as compared to control mice which received anti-bovine serum albumin (BSA) sera. Antitoxin had no protective effect on mice challenged with nontoxigenic strains of *P. aeruginosa*.

Similar results have also been obtained by actively immunizing mice with formalin inactivated highly purified (LPS-free) exotoxin A (f-toxoid) (Pavlovskis *et al.*, 1981). Three or four immunizations with the toxoid (10 µg per dose), prepared according to the procedure of Abe *et al.* (1978), and adjuvant resulted in high levels of antibody, an increase in $LD_{50}$ (Table 3) and a significant increase in their survival rate (50–85%) (Table 4, Fig. 1) of infected

**Table 3** Mean lethal doses for immunized[a] mice infected with *P. aeruginosa*

| Infecting strain | Mean lethal dose ($log_{10}$) | |
|---|---|---|
| | Control group[b] | Immunized group |
| PA 103 | 4.75[c] | 5.55 |
| | 4.73[c] | 5.93 |
| PA 220 | 0.23[c] | 2.03 |
| | 1.76[c] | 3.36 |

[a] Mice immunized with f-toxoid.
[b] Control mice immunized with formalinized BSA.
[c] $P<0.01$; two-tailed Student "t" test.

**Table 4** Survival of immunized[a] mice infected with *P. aeruginosa*

| Infecting strain | Toxin produced | Mortality[b] | | *P* |
|---|---|---|---|---|
| | | Controls[c] | f-TXD | |
| PA 103 | + | 12/15 | 3/14 | <0.01 |
| | | 14/15 | 1/7 | <0.01 |
| PA 103-29[d] | – | 15/15 | 15/15 | >0.05 |
| PA 220 | + | 14/15 | 3/9 | <0.01 |
| | | 13/15 | 7/15 | <0.01 |

[a] Mice immunized with f-toxoid (F-TXD).
[b] Number of dead mice/number total mice.
[c] Control mice immunized with formalinized BSA.
[d] PA 103-29 isolated and characterized: Ohman *et al.* (1980c).

mice compared with infected controls (<20%) immunized with formalinized BSA plus adjuvant. No significant differences in survival between toxoid immunized mice and their respective controls were seen when infected with nontoxigenic strains (Table 4), indicating that the protection afforded by f-toxoid is exotoxin A specific. Immunization with a glutaraldehyde-inactivated exotoxin A (g-toxoid) (Leppla *et al.*, 1978b) significantly increased the survival *time* of the toxoid immunized mice, but the survival *rate* was increased only to about 30% above the controls (Fig. 2) (Pavlovskis *et al.*, 1981). The protection of immunized mice could be improved when combined with gentamicin treatment (Figs 1 and 2) (Pavlovskis *et al.*, 1981). Virtually 100% survival was

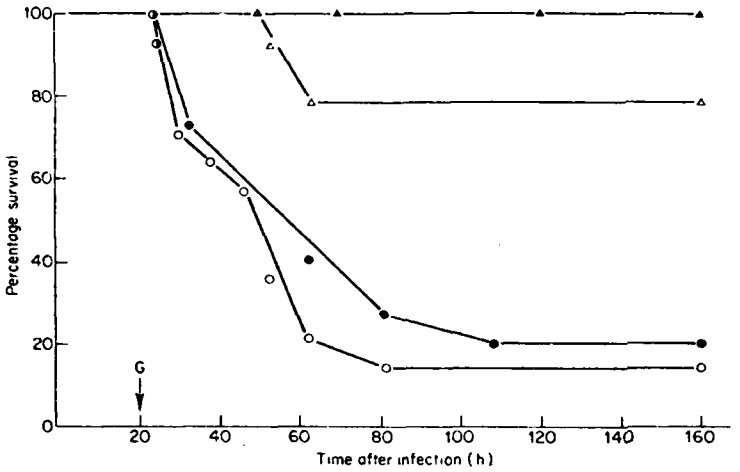



*Fig. 1* Survival of burned, infected mice immunized with f-toxoid (▲, △) and controls immunized with formalinized bovine serum albumin (●, ○). Mice treated with gentamicin (●, ▲); not treated with gentamicin (○, △). G = gentamicin injection. From Pavlovskis *et al.* (1981), reprinted by permission of the American Society for Microbiology.

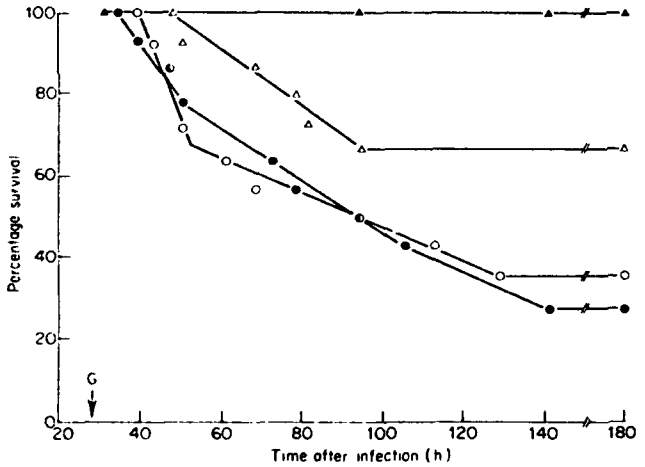


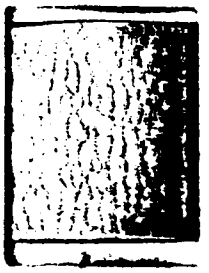

*Fig. 2* Survival of burned infected mice immunized with glutaraldehyde-inactivated exotoxin A (▲, △) and controls immunized with glutaraldehyde treated bovine serum albumin (●, ○). Mice treated with gentamicin (●, ▲); not treated with gentamicin (○, △). G=gentamicin injection. From Pavlovskis *et al.* (1981), reprinted by permission of the American Society for Microbiology.

obtained when immunized mice (f- or g-toxoid) were given a single dose of gentamicin 18–20 hours post-infection. At this time gentamicin had no protective effect on the survival of control mice. Thus it appears that during the early phase of infection the immunized host effectively neutralizes the toxic effects of exotoxin A. If at approximately 20 hours post-infection the organisms are eliminated and *in vivo* exotoxin A production ceases, the host survives. In the control-BSA immunized mice, however, exotoxin A was able to continuously exert its toxicity, and by 20 hours irreversible damage had occurred and the host, regardless of the presence or absence of the organisms, died.

Cryz *et al.* (1981) have studied various toxoiding procedures of exotoxin A in detail and have produced several suitable toxoids which elicit high antitoxin levels and protect mice. They have found that formalin induces structural alterations in a region of exotoxin A molecule which is essential for cytotoxicity but distinct from a site required for enzyme activity and thus enzymatic activity was not affected. In contrast, the addition of lysine to the formalin–toxin mixtures completely destroyed enzymatic activity within 48 hours. Upon storage the formalin-derived toxoids underwent partial toxic reversion, whereas the formalin–lysine toxoid did not.

Although exotoxin has been demonstrated in the tissues of animals infected with *Pseudomonas*, circulating exotoxin A in serum has not been unequivocally demonstrated in human patients. However, as pointed out earlier, in human patients, antibody to exotoxin A has been shown to rise with *Pseudomonas* bacteremia (Pollack *et al.*, 1976) and survival has been correlated with high antitoxin titres (Cross *et al.*, 1980; Pollack and Young, 1979). Cross *et al.* (1980) showed that patients who survived bacteraemia had six times the level of antitoxin as those who died from the bacteraemia. All patients with nonbacteraemic *Pseudomonas* infections had antitoxin, and there were no deaths attributable to *P. aeruginosa*. Cross *et al.* (1980) further demonstrated that death from *P. aeruginosa* bacteraemia was significantly associated with exotoxin A production by infecting strains. Of 11 infected patients who did not produce antibodies either to exotoxin A or LPS, seven of eight died when the infecting strain produced exotoxin A. In contrast, none of three died when the infecting strain was nontoxigenic ($P<0.001$).

The data presented indicates that exotoxin A both *in vitro* and *in vivo* acts in a highly specific manner and is extremely toxic. Observations from experimental animal infections and indirect evidence obtained from human patients strongly suggests that exotoxin A is an important virulence factor and that it contributes significantly to the pathogenic sequence of *P. aeruginosa* infections.

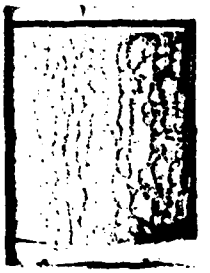


## III. PROTEASES

One characteristic of *P. aeruginosa* is its ability to liquefy gelatin or digest casein. This property has been known to microbiologists for more than 80 years. However, the first indication that protease may contribute to the pathogenicity of the bacteria came in 1958, when Fisher and Allen described a cornea-destroying *Pseudomonas* proteolytic enzyme. Morihara (1957) was first to characterize a *Pseudomonas* protease from a strain of *P. myxogenes*, later identified as *P. aeruginosa*. The observations by Liu (1969a) that *Pseudomonas* proteases caused haemorrhagic and necrotic skin lesions were further indications that these enzymes might act as virulence factors. Most strains of *P. aeruginosa* produce several proteases including those with elastolytic activity (Morihara, 1964; Kreger and Griffin, 1974; Wretlind and Wadstrom, 1977). Two of these enzymes, elastase and alkaline protease, have been characterized and studied by several investigators.

### A. Elastase

Morihara *et al.* (1965) purified and characterized a protease with elastase activity. Several investigators have confirmed Morihara's results (Balke and Sharmann, 1974; Jensen *et al.*, 1980a; Kreger and Gray, 1978; Kreger and Griffin, 1974; Scharmann and Balke, 1974; Wretlind and Wadstrom, 1977). The elastase (synonyms: protease II, protease fraction II, protease 1) can be separated from other proteases by ion exchange chromatography or isoelectric focussing and purified by ammonium sulphate precipitation or gel chromatography. More recently, affinity chromatography methods have been described (Morihara and Tsuzuki, 1975; Nishino and Powers, 1980). A completely synthetic medium for elastase production has been developed (Jensen *et al.*, 1980c). The medium contained glucose, glutamate, phenylalanine, valine and salts. Zinc and iron were essential for elastase production. Alkaline protease was not produced in this medium. The enzyme is a neutral, chelator-sensitive proteinase containing zinc (0.9 moles per molecule). It is active against casein, haemoglobin, elastin, fibrin and other proteins, but inactive or only weakly active against collagen. Elastase has a specificity for hydrophobic or bulky amino acids at the amino side of the splitting point (Morihara, 1974; Nishino and Powers, 1980). The molecular weight has been reported as 33 000–39 000. Sephadex chromatography gives significantly lower estimates (23 000), probably because of the interaction between the enzyme and the gel matrix resulting in retardation of the enzyme in its passage through the column. Its isoelectric point has been reported as lying between 5.7 and 6.6 (Morihara *et al.*, 1965; Kreger and Griffin, 1974; Balke and Scharmann, 1974; Wretlind and Wadstrom, 1977). The variation may be the result of autodigestion during purification.



Elastase is inhibited by chelating agents (EDTA, *o*-phenantroline), heavy metal ions, plasma alpha$_2$-macroglobulin, and phosphoramidon (Holder and Haidaris, 1979; Morihara and Tsuzuki, 1978).

Elastase is produced as a cell-associated inactive proenzyme early in the growth cycle and accumulates in the periplasmic space. It is converted to active enzyme *in vivo* by limited proteolysis either by alkaline protease produced by the organism or the elastase itself (Jensen *et al.*, 1980b). Yields of elastase and other exoproteins were decreased with increasing concentrations of iron in the growth medium (Bjorn *et al.*, 1979b).

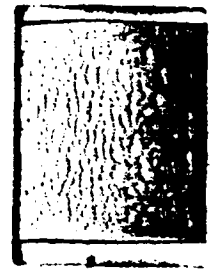
## B. Alkaline protease

Alkaline protease (Morihara, 1963) (synonyms: protease III, protease 2, *P. aeruginosa* proteinase) is produced by most *P. aeruginosa* strains. *In vitro* production, however, is repressed by free amino acids (Cryz and Iglewski, 1980; Morihara, 1964; Wretlind and Wadstrom, 1977). Alkaline protease was not produced in the defined medium described by Jensen *et al.* (1980c) in spite of good yields of elastase. This further emphasizes the differences in the regulation of the production of these enzymes. The molecular weight of alkaline protease is 48 400 (Inoue *et al.*, 1963; Morihara and Tsuzuki, 1977) and its isoelectric point 4.1–5.0. Alkaline protease contains 1–2 atoms of calcium per molecule. Cobalt ions have been shown to promote hydrolysis (Morihara and Tsuzuki, 1964, 1974).

## C. Other proteases

Another protease (neutral protease, protease fraction I, protease I) with an isoelectric point of 8.5 and an optimum pH of 6.5 has been identified in culture fluids (Kreger and Griffin, 1974; Morihara, 1964; Wretlind and Wadstrom, 1977), however, since the enzyme is produced in small amounts, it has not yet been purified. In contrast to elastase and alkaline protease this enzyme is not inhibited by EDTA.

Several investigators have described *Pseudomonas* enzymes with collagenase activity (Schoellmann and Fisher, 1966) or collagenolytic activities of *Pseudomonas* elastase (Carrick and Berk, 1975; Jensen *et al.*, 1980a), but others have been unable to detect collagenolytic activities among *P. aeruginosa* strains (Kreger and Griffin, 1974; Morihara and Tsuzuki, 1977; Wretlind and Wadstrom, 1977). This may be due to differences in the assay methods used by the various groups. It should be noted that although few reports indicate that *Pseudomonas* proteases may possess a weak collagenolytic activity, a collagenase similar to the enzyme produced by *Clostridium histolyticum* has not been demonstrated.

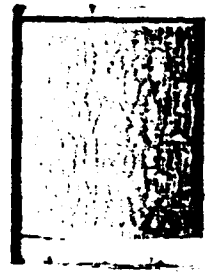


## D. Biological properties of proteases

The reported $LD_{50}$ values for elastase have shown a variation 60–400 μg per mouse) depending on the preparation and the route of administration (Kawaharajo *et al.*, 1975b; Meinke *et al.*, 1970; Wretlind and Wadstrom, 1977). The enzyme is most toxic when administered by intrapleural or intrapulmonary routes ($LD_{50}$ $<100$ μg); it is not lethal when injected subcutaneously. On a weight basis, *P. aeruginosa* exotoxin A is approximately 1000 times more lethal. Elastase is inhibited by the serum protease inhibitor alpha$_2$-macroglobulin, which may explain its low toxicity (Holder and Haidaris, 1979). Injections of purified elastase into animals usually leads to haemorrhage of internal organs. *In vitro* treatment of rabbit alveolar macrophages with elastase leads to agglutination and vacuolization (Leake *et al.*, 1978). However, *in vitro* elastase treatment does not damage the cytoplasmic membranes of HeLa cells or human diploid embryonic lung fibroblasts (Wretlind and Wadstrom, 1977). The observed changes are similar to those produced by trypsin.

Intrapulmonary and intrapleural injections of purified proteases caused extensive lung damage, with haemorrhages and necrosis of alveolar septal cells similar to that reported in cases of *Pseudomonas* pneumonia (Gray and Kreger, 1979; Meinke *et al.*, 1970; Kawaharajo *et al.*, 1975b; Shimizu *et al.*, 1974). Thus, the results indicate that *in vivo* protease production may be important in eliciting lung damage. Elastase and alkaline protease cause dermonecrosis after injection of less than 10 μg of purified enzyme, suggesting a role in wound and skin infection (Kawaharajo *et al.*, 1975a; Wretlind and Wadstrom, 1977).

Kreger and Griffin (1974) have demonstrated three cornea-damaging proteases in culture supernates of strains capable of causing kerititis. Less than 1 μg of these enzymes caused extensive colliquative necrosis of rabbit corneas after intracorneal injections. The necrosis progressed to descemetocele formation and corneal perforation. Light and electron microscopy examination of cornea showed degradation of the proteoglycan, but not of collagen fibrils (Brown *et al.*, 1974; Kawaharajo *et al.*, 1974; Kessler *et al.*, 1977b; Kreger and Gray, 1978). Several plasma proteins have been shown to be substrates for *Pseudomonas* proteases. Fibrin and fibrinogen as well as other coagulation factors are degraded *in vitro* by the proteases, and this probably explains the haemorrhages observed after the injection of purified enzymes into animals (Scharmann and Kraft, 1974a). Elastase also inactivates *in vitro* several complement factors (C1, C3, C5, C8, and C9) (Schultz and Miller, 1974). Inactivation of human alpha$_1$-proteinase inhibitor has also been reported (Morihara *et al.*, 1979). If this does occur *in vivo*, the loss of the protease inhibitor could permit leucocyte serine proteases to cause tissue damage.



## E. The role of proteases in infections

*P. aeruginosa* infections of the eye are not common, but when they do occur they often result in loss of vision of the infected eye. Since the corneal proteoglycan is a sensitive substrate for *Pseudomonas* proteases, it has been suggested that these enzymes are the main cause of tissue damage. Experimental infections show a loss of proteoglycan ground substance with dispersal of undamaged collagen fibrils (Gray and Kreger, 1975). Similar observations have been made after intracorneal administration of proteases (Kessler *et al.*, 1977b; Kreger and Gray, 1978). Protease-producing strains have been shown to cause experimental keratitis in mice, whereas protease negative strains are avirulent (Kawaharajo and Homma, 1978). Both passive and active immunization against elastase, alkaline protease, and endotoxin-protein (OEP) protected against experimental eye infections in mice (Hirao and Homma, 1978). Recently, Ohman *et al.* (1980b) studied the role of elastase in corneal infection in mice using a mutant that produced an altered elastase due to mutation in the structural gene of the enzyme. The elastase mutant (PA01-E64) was as virulent as the wild type strain (PA01). The mutant, however, did produce alkaline protease which also has cornea-damaging activity. It should be pointed out that other *Pseudomonas* products such as exotoxin A (Iglewski *et al.*, 1977a) and heat-stable haemolysin (Johnson and Allen, 1978) may also contribute to corneal damage. It has also been suggested that host-derived proteases and collagenase may contribute to the pathogenesis of *Pseudomonas* keratitis (Kessler *et al.*, 1977a; Van Horn *et al.*, 1978).

*Pseudomonas* pneumonia is characterized by intra-alveolar haemorrhage, necrosis of alveolar septal cells, and infiltration of mononuclear cells suggestive of protease-induced damage (Gray and Kreger, 1979; Nordstoga, 1968; Shimizu *et al.*, 1974; Tillotson and Lerner, 1968). Sera from human patients with *Pseudomonas* pneumonia (Homma *et al.*, 1975) and cystic fibrosis (Klinger *et al.*, 1978) contained antibodies against elastase and alkaline protease. Klinger *et al.* (1978) was able to demonstrate an inverse correlation between antibody titres and the clinical severity of the disease. A vaccine containing toxoids of elastase, alkaline protease and OEP protected mink against haemorrhagic pneumonia (Aoi *et al.*, 1979; Homma *et al.*, 1978).

A role for *Pseudomonas* proteases in burn infection was suggested by Carney *et al.* (1973). Kawaharajo and Homma (1977), using an experimental protease–elastase and OEP toxoid vaccine, were able to protect mice against experimental burn infection. Snell *et al.* (1978) and Holder and Haidaris (1979) reported that injections (10 μg) of elastase, alkaline-protease or *Bacillus thermoproteolyticus* protease (thermolysin) together with protease deficient *Pseudomonas* strain (PA 103) in burned mice caused a 1000-fold reduction in $LD_{50}$. This effect was specific for strains of *P. aeruginosa* since the $LD_{50}$ of other pathogenic bacteria was unaffected by the injection of

proteases. The protease inhibitor alpha$_2$-macroglobulin gave a significant protection against protease positive strains (Holder, 1981).

The *in vivo* role of a product of an organism can best be studied by using mutants lacking the ability to produce that particular product. This approach has been used to define the virulence factors of salmonellas and *Staphylococcus aureus* in experimental animal infections (Forsgren, 1972; Lindberg *et al.*, 1974). Pavlovskis and Wretlind (1979) studied the role of elastase in mouse burn infections using two protease-deficient mutants of a protease-positive clinical strain, PAKS-1. Both mutants were defective in the formation of extracellular proteases and several non-toxic exo-enzymes, but produced exotoxin A. In the burned mouse model (Stieritz and Holder, 1975) the $LD_{50}$ of the mutants were one $\log_{10}$ higher than that of the wild-type strain (Table 5) (Pavlovskis and Wretlind, 1979). The addition of 5–45 μg purified elastase to the infecting inoculum of elastase-deficient mutants reduced survival time and the number of surviving mice. Passive immunization with rabbit anti-elastase serum gave significant protection against the elastase-producing wild-type strain but not against the elastase-deficient mutants. Anti-elastase serum also prolonged survival time of mice infected with an elastase-deficient mutant and purified elastase. Quantitative blood cultures in mice infected with 1 $LD_{100}$ of protease-producing PAKS-1 or its protease-deficient mutants showed that the mice infected with protease-deficient strains had lower viable bacterial counts than mice infected with PAKS-1. However, when burned mice were infected with a mixture of PAKS-1 and one of the mutants at a 1:1 ratio, the number of protease-deficient organisms in the blood was considerably higher than in infections with the mutants alone.

The role of elastase in experimental burn infections was further studied in

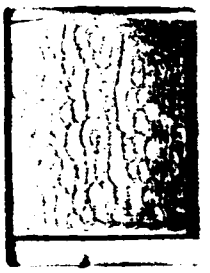
**Table 5** Virulence of elastase-producing *P. aeruginosa* strains and their elastase-deficient mutants[a] in mouse burn infections

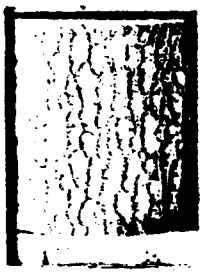
| Strains | Elastase | $\log_{10}$ $LD_{50}$[b] |
|---|---|---|
| PAKS-1 | + | 3.8 |
| PAKS-10 | (+)[c] | 4.8 |
| PAKS-17 | − | 4.9 |
| PAO1 | + | 4.1 |
| PAO1-E64 | − | 5.1 |

[a] PAKS-10 and PAKS-17 characterized: Pavlovskis and Wretlind (1979); PAO1-E64: Ohman *et al.* (1980b).
[b] $P<0.02$.
[c] Weakly elastase positive.

our laboratory (unpublished data) by using a mutant that produced an altered elastase. Ohman and associates (Ohman *et al.*, 1980b) isolated a mutant that produced a temperature-sensitive elastase, probably because of a mutation in the structural gene for elastase. The $LD_{50}$ for this mutant was one $\log_{10}$ higher than for the parent strain (Table 5). These data are in good agreement with the results from the study by Pavlovskis and Wretlind (1979) described above.

The data presented indicate that proteases, and elastase in particular, may contribute to the pathogenicity of the organism. The mechanism by which proteases exert their toxic action during an infection is not clear. Because of their high $LD_{50}$ it is unlikely that proteases alone are responsible for the lethal effect of *P. aeruginosa*. The results of Pavlovskis and Wretlind (1979) suggest that elastase may be important in overcoming the host's initial defence mechanisms. This may be either as the result of proteolytic action which provides additional nutrients for the invading bacteria as suggested by Cicmanek and Holder (1979) or by the destruction of the anatomical barriers preventing the spread of the organisms. The suggestion that protease is important in overcoming the host's defences is also indirectly supported by the work of Wretlind and Kronevi (1978), who infected cyclophosphamide-treated mice with protease-positive or -negative mutants. In contrast to the work of Pavlovskis and Wretlind (1979) they found no differences in $LD_{50}$ between the strains, nor did they find any differences in $LD_{50}$ when protease-positive and -negative strains were injected with mucin in normal mice. Both models, however, were designed to overcome the host's initial defence mechanisms, thus negating any advantage a protease-producing strain might have. The cyclophosphamide treatment reduced the leucocyte count from approximately $8000\,mm^{-3}$ for normal mice to less than $500\,mm^{-3}$ for cyclophosphamide-treated mice (Pavlovskis and Wretlind, 1979). In the second model, the mucin protected the organisms and increased the sensitivity of the host to the invading organisms. Once the infection is established, however, proteases may contribute to local tissue damage. B. H. Iglewski, for example, found elastase in tissue homogenates from *Pseudomonas*-infected mice (personal communication). The role of proteases in established septicaemia is probably only marginal. In the case of *Pseudomonas* eye infections proteases are capable of degrading corneal proteoglycan ground substance and are responsible for the resulting structural alterations. Proteases are considered to be the cause of the rapid invasion and corneal dissolution. It has been similarly shown that proteases produced by *P. aeruginosa* cause dissolution of pulmonary tissue in rabbits. Both passive and active immunization of experimental animals as well as treatment with protease inhibitor alpha$_2$-macroglobulin offers a certain degree of protection against *P. aeruginosa* infections. Thus the data indicate that *Pseudomonas* proteases contribute to the pathogenic mechanism of the organism.





## IV. MEMBRANE-ACTIVE TOXINS

### A. Haemolysins

Most *P. aeruginosa* strains produce two haemolytic substances. One is heat-labile and is probably phospholipase C with lecithinase activity (Esselmann *et al.*, 1961; Liu, 1966a; Wretlind *et al.*, 1973) (see below); the other is a heat-stable glycolipid composed of rhamnose and β-hydroxydecanoic acid (Hisatsuka *et al.*, 1971; Jarvis and Johnson, 1949; Sierra, 1960). The phosphiolipase and the glycolipid are usually produced together in an environment low in phosphate and high in carbohydrate content (Liu, 1964). Recently a purified haemolysin preparation containing two haemolytic glycolipids has been reported (Johnson and Boese-Marrazzo, 1980). The haemolytic glycolipid is a detergent capable of solubilizing phospholipids (Hisatsuka *et al.*, 1971; Kurioka and Liu, 1967a). Its effect on cell membranes is also detergent-like (Thelestam and Mollby, 1979). Methods for production and purification of the haemolytic glycolipid have been described (Berk, 1964; Johnson and Boese-Marrazzo, 1980). It appears that the glycolipid, at least *in vitro*, may enhance the activity of the phospholipase (Kurioka and Liu, 1967a, 1967b).

The glycolipid is relatively non-toxic. Given intraperitoneally, its $LD_{50}$ is approximately 5 mg for mice (Jarvis and Johnson, 1949) and its haemolytic activity is inhibited by serum albumin (Berk, 1964). Thus, a significant role for the glycolipid in *Pseudomonas* infections is unlikely. Johnson and Allen (1978) have presented evidence suggesting that glycolipid could contribute to tissue damage in *Pseudomonas* keratitis due to enzyme release from leucocytes. Al-Dujaili (1976) reported that *P. aeruginosa* strains isolated from the respiratory tract of patients were more strongly haemolytic than strains isolated from other sources, suggesting a role for the glycolipid in lung infections.

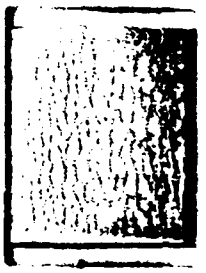
Although evidence indicated that the heat-labile haemolysin is phospholipase (synonyms: lecithinase, heat-labile haemolysin, haemolysin A) no direct proof of their identity had been established (Liu, 1974). Recently a heat-labile haemolysin (fraction A) was purified by isoelectric focussing and sucrose density-gradient which apparently is identical to phospholipase C (Watanabe *et al.*, 1978). This protein was shown to hydrolyse lecithin to produce phosphorylcholine (phospholipase C activity) and did not require any cofactors for its haemolytic activity. The haemolytic and phospholipase activities could not be separated indicating that the haemolytic protein is a phospholipase. Berka *et al.* (in preparation) have effectively purified *Pseudomonas* phospholipase C from low phosphate culture supernatants by adsorption with lecithin affinity gel and Sephadex G-75 gel filtration, resulting in approximately 940-fold purification. The $K_m$ value for the enzyme was

established to be 167 μM, and $V_{max}$ to be $-2$ nmol min$^{-1}$. The isoelectric point was near 5.9, in good agreement with the value of 5.75 measured by Watanabe (1978) (Berka and Vasil, personal communication). When Berke *et al.* analysed the phospholipase by SDS-PAGE, they found that areas corresponding to enzyme activity stained diffusely with Coomassie Blue and fuschin-bisulphite suggesting that phospholipase C was either a large glycoprotein or was associated with a heterogeneous carbohydrate, possibly LPS.

The *in vitro* production of phospholipase appears to be regulated by end-product repression (Johnson and Allen, 1978). Enzyme secretion required a carbon source and ammonium, potassium or calcium ions (Stinson and Hayden, 1979) and it was repressed by inorganic but not organic phosphates (Liu, 1974; Stinson and Hayden, 1979). The calcium requirement could be substituted by magnesium or strontium ions. Gray *et al.* (in preparation) have suggested that since phospholipase C and alkaline phosphatase co-purify on the lecithin affinity column, a functional relationship to liberate inorganic phosphate from phospholipids may exist between the two enzymes. They have also found that alkaline phosphatase and phospholipase C are co-ordinately depressed by inorganic phosphate starvation.

Although phospholipase C possesses a number of toxic properties, the discussion concerning its role in pathogenesis has remained largely speculative. When phospholipase C preparations, free of protease activity, were injected into the skin of rabbits or guinea pigs, they produced, between 24–48 hours, necrosis of an area with a central abscess surrounded by a zone of erythema (Liu, 1966a). Intraperitoneal injections of the enzyme resulted in hepatic necrosis. The haemorrhagic lesions frequently observed in internal organs following protease administration have not been observed with phospholipase. However, attempts to demonstrate *in vivo* production of phospholipase in experimental animals have not been successful (Liu, 1966b), nor could phospholipase be produced *in vitro* when rabbit serum was used as source of nutrients (Liu, 1964).

The alveolar surfaces of lungs are covered with surfactants, made up mostly of lipids. Reynolds and Fick (1979) have suggested that phospholipase has the potential to destroy lung surfactants and lung tissue, thus possibly causing bronchiectasis and atelectasis. It is conceivable that the surfactants which contain phospholipids induce production of phospholipase. *P. aeruginosa* strains which produced *in vitro* significant amounts of lecithinase (phospholipase C) ($34.5 \pm 6.7$ μg ml$^{-1}$) when administered intranasally (via aerosol) to mice were able to multiply in the lung and were not cleared as rapidly as strains which were not excellent lecithinase producers ($8.86 \pm 1.2$ μg ml$^{-1}$) (Southern *et al.*, 1970). Berka and Vasil (in preparation) found that urinary tract isolates consistently produced highest amounts of phospholipase C as compared to



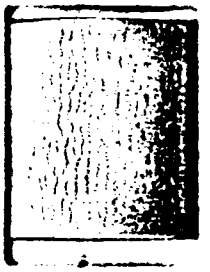
other clinical isolates. Lung and sputum isolates also produced high levels, but greater variability existed among strains. Blood and wound isolates produced significantly lower amounts. The data suggest that alterations of pulmonary surfactants may be one factor enabling the organism to invade and colonize the lung. Baltch *et al.* (1979) reported that blood culture isolates produced greater quantities of phospholipase C and proteases than non-bacteraemic isolates. However, they were unable to show any correlation between the *in vitro* quantity of phospholipase and the prognosis of these patients. Their study suggested a local rather than systemic importance of phospholipase C in the pathogenesis.

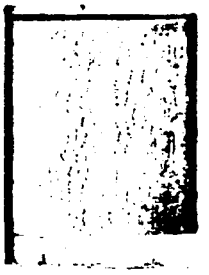
Phospholipase C probably is not an important factor contributing to the lethality of *Pseudomonas* infections. Experimental and clinical observations suggest that phospholipase-producing strains may have an added advantage in invading the host's tissues and thus facilitate blood stream invasion. There is no statistical correlation between death or survival of experimental animals or patients and the phospholipase produced by the invading organisms.

## B. Leucocidin

Scharmann (1976a, 1976b, 1976c, 1976d; Scharmann *et al.*, 1976) has characterized a cytotoxic protein active against leucocytes. This toxin designated leucocidin appears to be distinct from other *P. aeruginosa* toxins. Leucocidin does not react with anti-exotoxin A serum, and the amino acid composition of the two toxins is also different (Lutz, 1979). The toxin was produced as a cell-bound inactive precursor and released upon cell lysis and activated by proteases. Good yields of leucocidin were obtained through autolysis of washed bacterial suspensions at 37°C for 56 hours. Only 4 of 110 strains tested produced detectable amounts of the protein (Scharmann, 1976c).

Leucocidin was purified from cell autolysate by ammonium sulphate precipitation and gel chromatography. It had a molecular weight of 27 000 daltons and isoelectric point of 5.0–5.2. The purified toxin was sensitive to pronase, but resistant to several other proteases such as *Pseudomonas* elastase, trypsin, pancreatic elastase, papain, subtilisin (Scharmann, 1976c). The leucocidin exhibited the same properties as many other cytolytic toxins from bacteria. It damaged granulocytes from various animal species and human lymphocytes. Leucocidin was also cytopathogenic for different tissue culture cells, but did not cause lysis of erythrocytes or isolated leucocyte granules. After exposure to leucocidin, granulocytes became round and motility was lost. Protoplasmic extrusions appeared on the cell membrane. The final stage showed an enlarged, rounded vesicle with apparently intact plasma membrane. The cytotoxic action was studied on bovine granulocytes by following



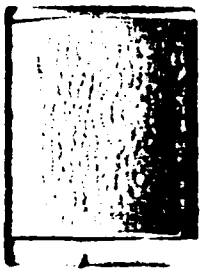


release of various intracellular markers (Scharmann, 1976a). Low-molecular markers ($K^+$, $^{86}Rb^+$, glucose) were lost from the granulocytes within 1-2 minutes after addition of leucocidin. The release of high molecular markers ($^{51}Cr$ bound to proteins) occurred only after swelling of the cells. Studies with [$^{125}I$]-labelled toxin indicated two binding sites, one at the surface of the plasma membrane, and one that became accessible to the toxin in the course of the cytotoxic action. In the presence of $Ca^{2+}$ the velocity of the toxin fixation was increased. The leucocidin receptor was probably an integral protein of the plasma membrane (Scharmann, 1976b). The leucocidin is relatively toxic, its $LD_{50}$ being about 1 μg per mouse (Scharmann, 1976d). Scharmann's findings have been confirmed in a more recent paper by Lutz (1979), who also demonstrated fatty liver necrosis in mice dying after injection of leucocidin. The toxin also caused cardiovascular failure in mice and rats (Frimmer *et al.*, 1976; Hegner *et al.*, 1976) and loss of potassium from perfused rat livers or isolated hepatocytes (Frimmer and Scharmann, 1975).

The role of leucocidin in *Pseudomonas* infections is not known. Scharmann (1976a) has suggested that the neutropenia found in *Pseudomonas* sepsis is caused by the action of this toxin. However, Sensakovic and Bartell (1974) reported that a toxic slime fraction caused leucopenia in mice, and the relative contribution of these factors and of exotoxin A to leucopenia remains to be determined. Finally, the low incidence of leucocidin producing strains argue against any significant importance of leucocidin in *Pseudomonas* infections.

## V. ENTEROTOXIN AND VASCULAR PERMEABILITY FACTOR

Part of the problem of studying the infectious aetiological agents of gastro-enteritis is the difficulty in identifying the pathogens among a large number of indigenous organisms. Thus, even though *P. aeruginosa* has been implicated in diarrhoeal conditions since the turn of the century (Williams, 1894) and a number of "diarrhoeas of unknown origin" have been attributed to the organism (Dold, 1918; Jellard and Churcher, 1967; Ensign and Hunter, 1946; South, 1971), its role in gastro-enteritis has not been clearly established. In 1971 the production of an enterotoxin by *P. aeruginosa* was demonstrated by its capacity to cause fluid accumulation in rabbit ileal loops following the injection of live organisms (Kubota and Liu, 1971). The amount of fluid accumulated was less than observed with *Vibrio cholerae*. Recently the isolation of enterotoxic *P. aeruginosa* strains which give positive ileal loop tests in piglets and rabbits have been reported by several laboratories (Baljer and Barrett, 1979; Shriniwas *et al.*, 1979). We have also examined in our laboratory the ability of several *Pseudomonas* strains provided by Dr S. C. Sanyal (Banaras Hindu University) to induce fluid accumulation in rabbit



ileal loops. We found, as did Kubota and Liu (1971), that the amount of fluid was considerably less than with *V. cholerae*. For *V. cholerae* the ratio of volume:length (approximately 8.0 cm) was between 1.0 and 1.2, whereas for the *Pseudomonas* strains it was between 0.25 and 0.47 (Merrell and Pavlovskis, unpublished data). Apparently the ability to induce fluid accumulation is very labile for *Pseudomonas* since it has already been lost in two out of four strains tested.

The enterotoxin has not been purified, but it is heat-sensitive and could be destroyed by the action of trypsin (Kubota and Liu, 1971). It appears to be distinct from any of the other toxic materials produced by *Pseudomonas* such as exotoxin A, haemolysin, or phospholipase. Okada *et al.* (1976) demonstrated a positive rabbit ileal loop test, when one-milligram quantities of purified *Pseudomonas* elastase or alkaline protease were injected into the loops. It seems unlikely, however, that proteases could be produced in such large quantities in the intestines during a natural infection.

Production of a vascular permeability factor (PF) by *P. aeruginosa* similar to the PF associated with *V. cholerae* enterotoxin has been reported (Kusama, 1974; Kusama and Huss, 1972; Shriniwas *et al.*, 1979). This factor was also destroyed by heating or treatment with trypsin and required a heat-stable cofactor for its activity. Strains producing PF tended to be more virulent in experimental mouse burn infections. The identity between PF and the enterotoxin described by Kubota and Liu (1971) has not been established. However, Shriniwas *et al.* (1979) have reported that enterotoxin-positive strains were always positive for PF suggesting that these two factors may be identical.

Because the *P. aeruginosa* enterotoxin has not yet been purified and characterized in any detail and its nature has not been established, it may be advisable to avoid the term *enterotoxin* and refer to it as a "rabbit ileal loop factor".

## VI. SLIME GLYCOLIPOPROTEIN

In recent years the role of slime in *Pseudomonas* infections has been studied. The function of slime in the pathogenesis of the organisms was first reported by Liu *et al.* (1961). Bartell *et al.* (1970) isolated and purified by relatively gentle procedures a toxic slime antigen (glycolipoprotein, GLP) which caused leucopenia and death after injection into mice.

Endotoxin-free GLP (MW > 100 000) was purified from the extracellular slime layer by extraction with saline, ethanol precipitation, ion exchange chromatography, and ultracentrifugation (Bartell *et al.*, 1970). Chemical analysis showed that GLP contained hexoses, hexosamines, uronic acid, lipid

and protein. Four fatty acids which were components of GLP were not present in LPS preparations from the same strains. The LPS contained one fatty acid not found in GLP (Sensakovic and Bartell, 1974). Antigenic differences between LPS and slime were demonstrated by immunodiffusion and indirect haemagglutination inhibition (Sensakovic and Bartell, 1974). Analysis of antigenic relatedness between GLPs from different strains by indirect haemagglutination inhibition demonstrated an antigenic diversity (Dimitracopoulos and Bartell, 1980). Homologous GLP showed the strongest inhibition while heterologous GLP was only slightly active. There was no correlation with the immunotype (Fisher *et al.*, 1969) of the strains. GLP also possessed receptor-like activity for certain phages (Bartell *et al.*, 1971) and the carbohydrate moiety appeared to be a substrate for phage depolymerase (Sensakovic and Bartell, 1975). *Pseudomonas* strains lysogenic for or resistant to phage 8 did not produce the depolymerase substrate and their GLPs differed quantitatively in neutral sugar, amino sugar, and protein content from the wild type GLP (Dimitracopoulos and Bartell, 1979). The GLP is distinctly different from the extracellular exopolysaccharide produced by "mucoid" strains of *P. aeruginosa* isolated predominantly from cystic fibrosis patients (Doggett *et al.*, 1964; Doggett and Harrison, 1972; Hoiby, 1977; Reynolds *et al.*, 1975). The exopolysaccharide is an alginic-acid like polysaccharide consisting mostly of D-mannuronic and L-glucoronic acids (Carlson and Matthews, 1966; Evans and Linker, 1973).

Injections of GLP into mice induced leucopenia and proved lethal. The $LD_{50}$ of the GLP for mice was 30 μg per g (IP) of body weight, whereas the $LD_{50}$ for LPS from the same strain was 60-90 μg per g body weight (Sensakovic and Bartell, 1974). *In vitro* GLP inhibited phagocytosis and exerted a mitogenic effect on human blood lymphocytes (Papamichail *et al.*, 1980; Sensakovic and Bartell, 1980).

When GLP was treated with phenol to remove the protein, the remaining fragment still possessed all the biological activities of GLP. After acetic acid treatment which removed all of the lipid and most of the protein, the remaining carbohydrate was not toxic but retained its antigenic specificity and its ability to inhibit phagocytosis. Fragments released after treatment with phage 2-depolymerase lacked all biological activities of GLP. These results indicate that the toxic activities (leucopenia, lethality) are associated with the lipid moiety and are not due to contaminants such as exotoxin A as previously suggested (Liu, 1974). The antigenic specificity and antiphagocytic activity appears to reside in the carbohydrate portions (Sensakovic and Bartell, 1975).

Leucocytes obtained from mice injected with GLP agglutinated with rabbit anti-GLP serum indicating an *in vivo* association between GLP and leucocytes. This observation was also supported by fluorescein-labelled antibody studies. *In vivo* studies suggested that purified GLP when injected into mice

enters the blood stream and becomes associated mainly with neutrophils and that the neutrophil-GLP complex is deposited in the liver leading to leucopenia (Lynn *et al.*, 1977). It appears that GLP is an important toxic product of the organisms.

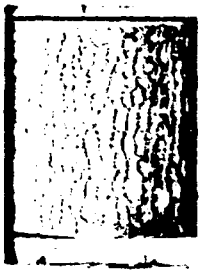
Active and passive immunization of mice against GLP protected from leucopenia and death after challenge with live organisms. Although there was some degree of cross-protection between strains, each antiserum protected most effectively against the homologous strain (Sensakovic and Bartell, 1974, 1977). LPS failed to absorb the protective antibodies from slime antiserum. The data suggest a pathogenic role for GLP.

## VII. EXOENZYME S

Recently, a second extracellular protein (exoenzyme S) with ADP ribosyltransferase activity produced by some strains of *P. aeruginosa* has been isolated and purified about 30-fold (Bjorn *et al.*, 1979a; Iglewski *et al.*, 1978). The medium used for exoenzyme S production is similar to that previously developed for exotoxin A production (Thompson *et al.*, 1980). In addition, exoenzyme S production required the presence of chelating agents such as nitrilotriacetate or EDTA, whereas exotoxin A production does not (Iglewski and Sadoff, 1979). However, EDTA inhibits the growth of the organism thus resulting in a lower overall yield. Attempts to produce exoenzyme S by growing the organism in chemically defined medium (DeBell, 1979) have not been successful (Iglewski, personal communication).

Exoenzyme S differs from exotoxin A in that it is heat-stable and does not ADP-ribosylate EF-2, but, rather, modifies one or more different proteins present in eucaryotic cell extracts. Also whereas urea and dithiothreitol treatment enhances enzymatic activity of exotoxin A, it destroys the activity of exoenzyme S. Serologically the two proteins do not cross-react. The enzymatic activities of exotoxin A and exoenzyme S cannot be neutralized by anti-S or antitoxin A respectively. The evidence suggests that exotoxin A and exoenzyme S are structurally different. It appears that in culture supernatants, exoenzyme S aggregates or associates with other proteins or lipoproteins (DeBell, personal communication; Thompson *et al.*, 1980). Sokol and associates (personal communication) detected *in vitro* exoenzyme S production in 38% of 124 clinical *P. aeruginosa* isolates from patients with bacteraemia or burn infections. Thus production of exoenzyme S by clinical isolates of *P. aeruginosa* is not a rare event. The majority of strains which produce exoenzyme S also produce exotoxin A, however, 11% of strains produced only exoenzyme S.

No studies have been reported regarding the toxicity or the role of



exoenzyme S in *P. aeruginosa* infections. Indirect evidence, however, does suggest that exoenzyme S may contribute to the pathogenicity of the organism. The enzyme is produced *in vivo* and in experimental infections (Bjorn *et al.*, 1979a). Skin extracts and sera from burned mice infected with a lethal dose of a non-toxigenic, exoenzyme-S-producing strain contained exoenzyme activity. Although this strain produces elastase, because of the organism's extreme lethality ($LD_{50} = 1.1 \times 10^2$) for burned mice, it is unlikely that elastase is completely responsible for its virulence. In human patients infections with exoenzyme S positive, exotoxin-A-negative strains frequently result in death (Cross *et al.*, 1980; Iglewski, personal communication). In one study, of the seven strains identified as producing exoenzyme S, five were from patients who died (Thompson *et al.*, 1980).

The role, if any, of exoenzyme S in clinical infections is not known. Data regarding the toxicity of exoenzyme *in vitro* and *in vivo* systems, its production by clinical isolates, and the protective effects of specific antibodies are still required to evaluate its relative importance and its role in the pathogenesis of the organism.

## VIII. CONCLUSION

A vast amount of information on the production, isolation and characterization of *Pseudomonas* enzymes and toxins has been accumulated during recent years, but there are still important gaps in our knowledge. Although several of these toxins have been shown to contribute to the pathogenicity of the organism and their mode of action is known, little is known of the interaction of these toxins with tissues and the sequence of the pathological events.

After release from the bacterial cell, the toxin may be transported by the blood stream throughout the body and cause systemic symptoms or it may diffuse in the surrounding area and damage primarily the local tissues. Both events probably occur. Since the toxins produced by *P. aeruginosa* are considerably less powerful than those of the classical toxin-mediated diseases such as botulism or tetanus, the local effects are probably the more important ones. In a recent review, Costerton (1979) presented evidence that in nature and, very likely, in the infected host, *Pseudomonas* usually does not exist as free-floating organisms but, rather, is attached to surfaces as microcolonies enclosed in a fibrous polysaccharide matrix. If this indeed is the case, the micro-organisms would have a very efficient mechanism by which to discharge their deleterious toxins. A "free-floating" bacterium is much more susceptible to the host's defences, and the enzymes and toxins are diluted and more easily neutralized or destroyed before reaching their targets. In contrast, toxins

produced by organisms growing in a dense microcolony remain sufficiently concentrated to penetrate and damage the surrounding tissue. In such a system the toxins need not be highly toxic to be effective. Furthermore, organisms growing in microcolonies are less susceptible to antibodies and antibiotics.

Because of the diversity of the *P. aeruginosa* toxins, the observed pathology in most cases cannot be attributed to a particular toxin. It appears that two of the extracellular toxins, elastase and exotoxin A, contribute significantly to the pathogenicity of the organism, whereas the role of others (phospholipase C, slime glycolipoprotein, leucocidin, exoenzyme S and the rabbit ileal loop factor) is not as clear. The proteases apparently enhance the ability of the bacteria to establish the infection, either by providing additional nutrients or by destroying anatomical barriers or by both. During the later stages of the infection the effect of exotoxin A predominates. Exotoxin A is secreted from the onset of the infection, causing a continued and irreversible damage. In experimental infections the primary organ to be affected is the liver, but in man only rarely is *Pseudomonas* infection associated with hepatic malfunction (Cross *et al.*, 1980). None the less, because the toxic action of exotoxin A – inhibition of protein synthesis – is a general biochemical function and because *in vitro* exotoxin A does not exhibit any specificity for a particular cell line, there is no reason to believe that its toxic action should be confined to only one tissue. During an infection the host is exposed to a continuous release of small amounts of exotoxin A, which may have additive biochemical and pharmacological effects on the surrounding tissues. It is therefore very probable that other organs and tissues are affected as well and that their eventual impairment contributes to the illness and death of the host. An important facet of the exotoxin A effect may be its interference with leucocyte function, as suggested by *in vitro* data showing that toxin is lethal for human macrophages derived from peripheral blood monocytes (Pollack and Anderson, 1978; Yamada *et al.*, 1977).

Experimental data show that elastase and exotoxin A elicit high levels of antibodies both in experimental animals and in patients. These results suggest that these proteins should be considered for use in a prophylactic *Pseudomonas* vaccine. Despite advances in chemotherapy and supportive treatment, *Pseudomonas* infections still constitute a major unresolved clinical problem. Several attempts to produce a vaccine from whole cells by empirical methods or from endotoxin have been made, but most of these preparations have been either too toxic or too ill-defined for use in humans. Moreover some of these antigens have the disadvantage of varying in type specificity. On the other hand, elastase and exotoxin A can be obtained in chemically pure form and do not vary with the strain type. Their biological activity can be inactivated without destroying immunogenicity. Both passive and active

immunization with elastase and exotoxin A antisera or antigens protect animals against *P. aeruginosa* infections. The results with animals have been very encouraging, but the usefulness of these toxoids in a human vaccine remains to be determined. The proof, of course, will rest in the prevention of the disease.

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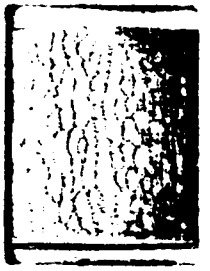

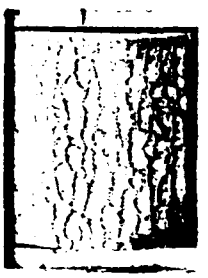

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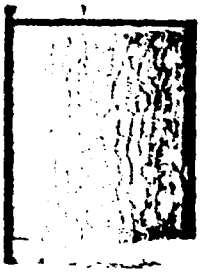

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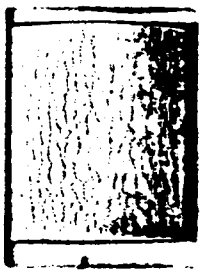

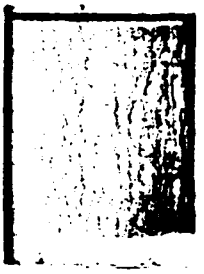

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